Structure
Previews

Pseudokinases: Functional Insights Gleaned from Structure

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Pseudokinases are an intriguing group inside the large protein kinase family. Lacking the highly conserved catalytic machinery, they can be still important for multiple functions in living cells. The structures of two pseudokinases VRK3 (Scheeff et al., 2009) and ROP2 (Labesse et al., 2009), presented in this issue of *Structure*, shed light on the internal machinery of these unique molecules. Structural information allows us to go beyond sequence alone in the analysis of these pseudokinases.

The human kinome contains over 500 proteins, most having splice variants, and these enzyme orchestrate and regulate much of the biology of the cell (Manning et al., 2002). As more protein kinase structures are solved, we are rapidly beginning to fill in the structural kinome, and this provides us with an opportunity to consider the structure, function, regulation, and evolution of these complex proteins in great depth. We also have come to appreciate that the protein kinases are not only catalysts but also scaffolds. The unique protein kinase fold has evolved not only to position many key residues for ATP binding and phosphoryl transfer, but also to expose chemically diverse surfaces to the solvent. These surfaces provide docking sites for many other proteins, including substrates, inhibitors, regulatory proteins and domains, and other signaling molecules. Included in the kinome are a number of kinases that were initially designated as "dead" kinases or "pseudokinases" because they lacked one or more essential conserved catalytic residues. The structures of two such pseudokinases are reported in this issue of *Structure*, VRK3 (Scheeff et al., 2009) and ROP2 (Labesse et al., 2009). VRK3 is a human vaccinia related kinase, while ROP2 is a virulence factor from *Toxoplasma gondii*. Another homolog of ROP2, ROP8, which was also designated as pseudokinase, was deposited in the Protein Data Bank earlier this year and is also discussed. Of particular interest to the signaling and structural biology communities is how to determine whether a putative pseudokinase, based on sequence alone, is truly inactive. Examining structures in addition to sequences can provide novel insights into to the functional relevance of evolutionary changes. Variation in conserved linear motifs is discussed very well (Scheeff et al., 2009); however, non-linear motifs that contribute to the global organization of a protein kinase molecule are not readily apparent from sequence. What do we learn from the structures of these three putative pseudokinases?

The kinase fold is characterized by two lobes, an amino-terminal lobe (N-lobe) and a carboxy-terminal lobe (C-lobe), with the adenine ring lying at the base of the cleft between the two lobes. The small lobe is mostly β strands while the C-lobe is mostly helical. A five-stranded β sheet of the N-lobe containing many of the conserved catalytic residues lies on the surface of the C-lobe at the active site cleft. The C-lobe is very stable and provides a docking site for other proteins, including substrates, while the N-lobe is quite malleable. Most of the conserved residues that contribute to substrate binding and catalysis cluster around the active site cleft; however, in the absence of nucleotide, the two lobes are uncoupled and the cleft is open. By having many protein kinase structures available—all with the same fold, it has been possible to define more precisely the architecture of the protein kinases, and several concepts are important to appreciate when one considers how a kinase is activated and whether it is capable of carrying out catalysis. Activation, typically achieved by phosphorylation of the activation segment in the C-lobe, leads to the organization of the active site, which is mediated by the formation of a hydrophobic regulatory (R) spine that couples residues in the N-lobe with residues in the C-lobe (Kornev et al., 2006). Because this spatially conserved motif is made up of hydrophobic non-linear residues, it was not recognized previously as a well-defined module. Further structural comparison of the kinase cores revealed that the αF helix serves as a scaffold for the organization of the entire molecule (Kornev et al., 2008). Both the R spine and a second hydrophobic spine radiate from the αF helix. The second spine is referred to as a catalytic (C) spine because it is completed by the adenine ring of ATP. Once the two lobes are thus coupled, the rest of the small lobe positions the γ-phosphate for transfer to a protein substrate, which is typically docked onto the surface of the C-lobe. How are the conserved residues clustered in the large and small lobes, and how do we establish unambiguously that a kinase is inactive? By analyzing the spines, these two kinase structures can immediately be classified into two different categories. VRK3 appears to be a genuine pseudokinase that is rigid and not able to bind ATP; it is thus cannot mediate any kind of catalysis. ROP2, based on the biophysical results presented by Labasse et al. (2009), also

is unable to bind ATP under these conditions, and thus appears to have no kinase activity. If ROP2 is to be active, it would have to utilize a completely unique mechanism, perhaps requiring other proteins or ligands.

If we only consider modification of conserved active site residues, it is difficult to say unambiguously that any given kinase will be inactive, since there are already several classic exceptions. A structure was required to reveal these novel adaptations. Wnk (With No K), for example, is a classic example in which the protein might have been considered to be a pseudokinase based on sequence alone because the lysine in β strand 3 was missing. Solving the Wnk structure, however, confirmed predictions that the function of the missing lysine was carried out by a lysine that filled the same space but was positioned in β strand 2 (Min et al., 2004; Xu et al., 2000). This creative juggling of conserved residues is seen frequently in the eukaryotic-like kinases (ELKs) that share the same catalytic mechanism as the eukaryotic protein kinases (EPKs) but lack the complex regulatory machinery and docking motifs in the C-lobe (Kannan et al., 2007). It should also be pointed out that mutation of this lysine does not necessarily abolish ATP binding; at least some kinases can still bind ATP perfectly well when the Lys is mutated, though they are catalytically impaired (Carrera et al., 1993). CASK is another example where the two key magnesium binding residues are missing. However, CASK was found to be an Mg^{2+}-independent kinase (Mukherjee et al., 2008); that is, it is actually inhibited by Mg^{2+}. CASK was also very specific for one substrate, neurexin. Thus, the absence of key conserved residues is not sufficient to rule out that the protein could not be active as a kinase under very specialized circumstances. In contrast to Wnk and CASK, VRK3 does appear to be a genuinely inactive kinase because it can no longer bind ATP, as the adenine pocket is filled. Additionally, ROP2 on its own seems unable to bind ATP, as described by Labesse et al (2009). A comparison of the spines demonstrates why this is so (Figure 1). Both VRK3 and ROP2 have an assembled R-spine so they represent a potentially "active" conformation. The catalytic spine in VRK3, however, is quite different from any other kinase that has been solved to date. VRK3 has three mutations in spine residues that actually fill in the adenine binding pocket and make it sterically impossible for any nucleotide to bind. Leucine from the β strand 7 (Leu^{173} in PKA) is replaced with a much bulkier phenylalanine, while valine from the β strand 2 (Val^{57} in PKA) is replaced with leucine in VRK3. The two parts of the spine are thus permanently fused to one another, making the closed configuration quite stable. The C-spine is further immobilized by the replacement of alanine in β strand 3 (A^{70} in PKA) with serine. The side chain OH of this serine interacts with backbone amide of L^{262} in the linker, thus causing its side chain to rotate around and face L^{180} and F^{313}. These three residues appear to form a solid hydrophobic knob that occludes the adenine binding pocket shutting down the kinase activity, since, to be active, the two lobes must be able to open and close. The fact that other key catalytic residues are missing is probably not particularly relevant since there is no longer any pressure to conserve them. VRK3 is almost certainly functioning solely as a rigid scaffold protein, and these modified residues, as well as other surfaces, may be important for this function. If VRK2 binds to the VHR phosphatase, for example, and renders it

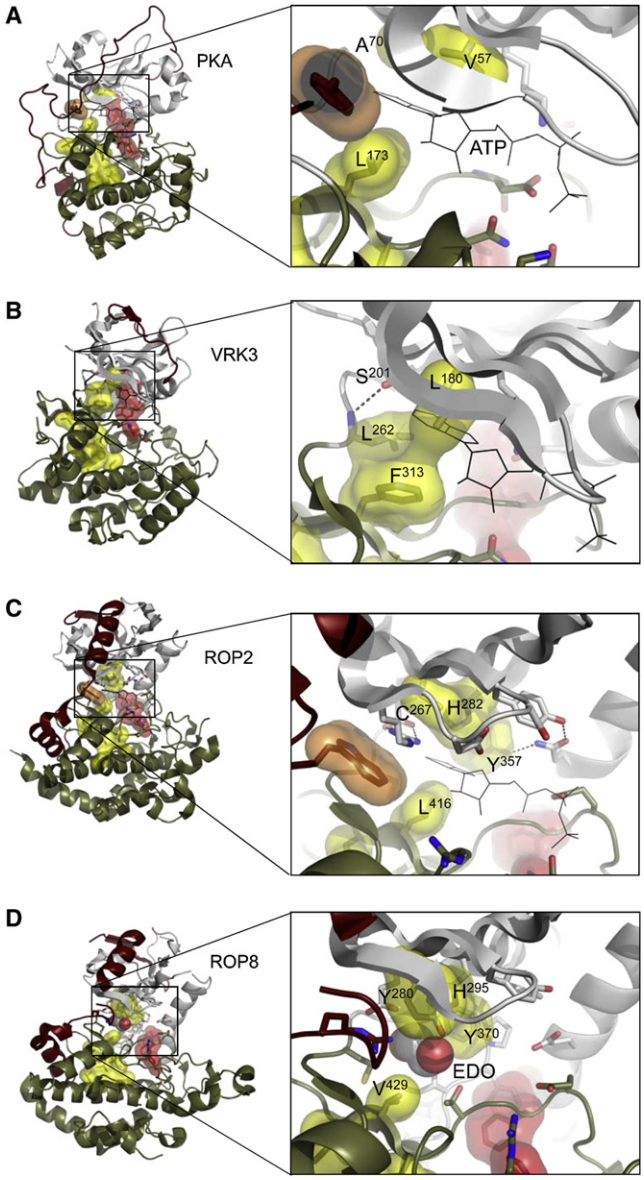

Figure 1. VRK3 Has a Novel and Rigid Catalytic Spine
In most protein kinases, the hydrophobic catalytic spine is completed by the adenine ring of ATP, which then couples the small and large lobes. Completion of the catalytic spine is shown here in three different protein kinases.
(A) In PKA (PDB ID 1ATP), as in all active eukaryote protein kinases, the C-spine is completed by the adenine ring of ATP.
(B) In "dead" kinases, such as VRK3 (PDB ID 2JII), the C-spine is fused together by large hydrophobic side chains. The adenine binding cavity is thus blocked. ATP, modeled from the PKA structure, is sterically prevented from binding.
(C) In ROP2 (PDB ID 2W1Z) the C-spine is not completed. The ATP molecule, modeled from the PKA structure (1ATP), has only minor steric clashes with the surrounding side chains.
(D) In ROP8 (PDB ID 3BYV) the C-spine is completed by a molecule of ethanediol (EDO).

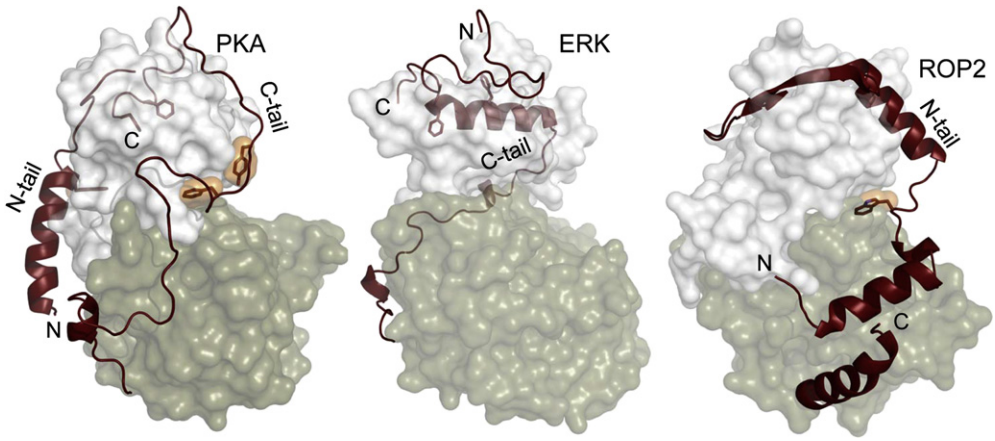

Figure 2. Flanking Tails Decorate the Conserved Structural Core in Various Ways, Regulating Their Function and/or Localization
Those flanking regions often have multiple functions that allow new surfaces to be exposed if the flanking segment is recruited elsewhere. We show here three examples: PKA (PDB ID 1ATP), ERK (PDB ID 2ERK), and ROP2 (PDB ID 2W1Z). PKA and ERK, in addition to being kinases and scaffolds for multiple proteins, including substrates and inhibitor proteins, are also themselves substrates for other heterologous kinases and for phosphatases. Thus, there are unlimited opportunities for kinases and pseudokinases to mediate protein-protein interactions.

active, then VRK3 may bind to this phosphatase and make it constitutively active. In any case, it is now important to identify the normal binding partner or partners for VRK3 and to also elucidate the partners for the homologous viral proteins that allow them to hijack the mammalian host.

ROP2 is interesting because it belongs to a family of proteins that play a key role in the cell invasion of *Toxoplasma gondii* into a host cell (Boothroyd and Dubremetz, 2008). Proteins in the ROP family typically have a C-terminal protein kinase domain. Some of these kinases appear to have all the criteria necessary to be active, whereas others such as ROP2 are missing several key residues. However, unlike VRK3, the adenine binding pocket is open and it could be envisioned that it potentially binds a bulky ligand. We also found that another ROP structure had been solved by the Structural Genomics Consortium and deposited in the Protein Data Bank (PDB ID: 3BYV). ROP8 has 73% sequence identity with ROP2 and adopts a very similar conformation (rmsd 1.2 Å). ROP8, however, also includes a molecule of ethanediol in the ATP adenine ring pocket and completes the C-spine. This may suggest that ROP2 could also accommodate an ATP molecule, but this statement awaits further analysis and investigation of ROP8, since analysis performed on ROP2 strongly supports the view that ATP-binding ability has been lost. No biophysical data is available for ROP8 at this time. The two ROP structures also highlight the importance of noncore residues. In both structures, the well-defined helical N-terminal tail folds over onto the core where it comes close to the ATP binding pocket (Figure 2). Labesse et al. (2009) demonstrate that N-terminal extension can have multiple functions. They demonstrate that the N-terminal tail can target the protein to membranes and function as a docking motif. As they point out, this is much more plausible than the previous suggestion that the αF helix serves as a transmembrane motif. When the N-terminus binds to a membrane, the hydrophobic surface of the core is also exposed and this could contribute further to docking to the membrane or it could bind to another protein. As seen in Figure 2, there are many other examples where the adjacent "tails" or domains contribute either positively or negatively to function. Diversity of the N terminus suggests that localization is, obviously, an important feature of this protein.

To understand the functional importance of pseudokinases, it is necessary to have structures to adequately interpret sequence variability. In addition, it will be essential to identify their binding partners. In the case of pseudokinases that are virulence factors, this will allow us to understand the mechanism whereby the virulence factors can hijack the host machinery and shut it down. Does it form a heterodimer with a host homolog? Does it bind to another protein? Does it adopt an unconventional mechanism for phosphorylation of itself or the partner? These are some of the challenges that the signaling and structural communities face.

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